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2 **PRKAR1A mutation causing pituitary-dependent Cushing**
3 **disease in a patient with Carney complex**
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Abstract

Context: Carney complex (CNC) is an autosomal dominant condition caused, in most cases, by an inactivating mutation of the *PRKAR1A* gene, which encodes for the type 1 alpha regulatory subunit of protein kinase A. CNC is characterized by the occurrence of endocrine overactivity, myxomas and typical skin manifestations. Cushing syndrome due to primary pigmented nodular adrenocortical disease (PPNAD) is the most frequent endocrine disease observed in CNC.

Case Description: Here we describe the first case of a patient with CNC and adrenocorticotrophic hormone (ACTH)-dependent Cushing disease due to a pituitary corticotroph adenoma. Loss-of-heterozygosity analysis of the pituitary tumour revealed loss of the wild-type copy of *PRKAR1A*, suggesting a role of this gene in the pituitary adenoma development.

Conclusion: *PRKAR1A* loss of function mutations can rarely lead to ACTH-secreting pituitary adenomas in CNC patients. Pituitary-dependent disease should be considered in the differential diagnosis of Cushing syndrome in CNC patients.

Introduction

Carney complex (CNC) is a syndrome characterized by spotty skin pigmentation, myxomas and endocrine abnormalities. Although several manifestations of this disease had been reported previously ¹, J. Aiden Carney and colleagues were the first to describe CNC as a distinct entity in 1985 ². Multiple lentiginos are the most common presenting feature, and frequently affect distinct areas such as the lips, the conjunctiva and the vaginal or penile mucosa ^{3, 4}. Myxomas can occur in various organs, particularly the heart, breast and skin. The most common endocrine disease observed in CNC is adrenocorticotrophic hormone (ACTH)-independent Cushing syndrome secondary to primary pigmented nodular adrenocortical disease (PPNAD) ⁴. Other CNC-related endocrine disorders include growth hormone (GH) excess and hyperprolactinemia secondary to pituitary tumours or hyperplasia, thyroid and testicular tumours, mostly represented by large cell calcifying Sertoli cell tumours (LCCSCT) ^{3, 4}.

In the majority of the cases, CNC is caused by inactivating mutations in the *PRKAR1A* gene (17q24.2) coding for the type 1 alpha regulatory subunit of the cAMP-dependent protein kinase A (PKA) ⁵. PKA plays a pivotal role in various cellular functions such as DNA replication, cell growth, proliferation and differentiation and is believed to act as a tumour suppressor gene via loss of the wild-type allele in affected tissues ^{6, 7}. Most of the previously described *PRKAR1A* mutations lead to premature stop codons and subsequent degradation of the mutant mRNA by nonsense mediated mRNA decay ⁸. Inactivating *PRKAR1A* mutations result in excess PKA signalling in affected tissues driving the tumorigenic process ^{7, 9}.

Case Presentation and Results

A 31 year-old man presented at our Endocrine Clinic with typical signs of Cushing syndrome. His physical exam revealed moon face, abdominal obesity, skin atrophy and pronounced red striae on the abdomen, upper arms and thighs; BMI was 27.1 kg/m². He had multiple junctional nevi, particularly on his torso. His previous medical history included surgical removal of the left testicle due to a large cell calcifying Sertoli cell tumour at the age of four and pilonidal sinus surgery at the age of 24. At the age of 25, the patient suffered a syncope while playing soccer. Echocardiography revealed myxomas in both atria with a 3×5 cm left atrial myxoma protruding through the mitral valve which necessitated surgical removal. His appearances and previous clinical history were highly suggestive of CNC ^{2, 3}.

The laboratory work up revealed markedly elevated urinary free cortisol (769 µg/24h, reference: 2.5-213.7 µg/24h) and increased basal ACTH (106 pg/ml, 23.3 pmol/l, reference: <46 pg/ml, <10.1 pmol/l; CLIA, Siemens Immulite2000). Morning serum cortisol was 11.4 µg/dl (314 nmol/l, reference: <1.8 µg/dl, 50 nmol/l) after a 1 mg overnight dexamethasone suppression test. Consecutive low and high dose dexamethasone suppression tests (4×0.5 mg dexamethasone daily for two days followed by 4×2 mg dexamethasone for two more days) did not result in adequate suppression of morning serum cortisol levels (basal: 24.2 µg/dl (668 nmol/L), after 48h: 14.0 µg/dl (386 nmol/l), after 96h: 19.0 µg/dl (524 nmol/l)). Corticotrophin releasing hormone (CRH) test failed to further stimulate his already elevated ACTH levels. The rest of his pituitary function tests were normal. These results were consistent with ACTH-dependent Cushing syndrome but not with primary pigmented nodular adrenocortical disease (PPNAD), a form of ACTH-independent Cushing syndrome commonly observed in CNC patients ^{3, 10}. Due to the lack of cortisol suppression

following the high dose dexamethasone suppression test and the absence of ACTH increase following CRH administration, we suspected ectopic ACTH production as the reason for hypercortisolism in this patient. Subsequent ^{18}F -fluorodeoxyglucose (^{18}F -FDG) positron emission tomography combined with computed tomography (PET/CT) as well as ^{18}F -dihydroxyphenylalanine (^{18}F -DOPA) PET/CT did not show any lesions that would have qualified as a source for ectopic ACTH production. Therefore, inferior petrosal sinus (IPS) sampling was performed and showed a significant central to periphery ACTH ratio as well as lateralization to the left (Table 1), strongly suggesting the pituitary as the origin of the autonomous ACTH production. Magnetic resonance imaging (MRI) of the sellar region showed a 4×6 mm left paramedian lesion, compatible with a pituitary microadenoma (Figure 1a,b), whereas MRI of the adrenal glands was unremarkable. The left paramedian pituitary tumour was resected by transsphenoidal surgery and histopathology confirmed a corticotroph adenoma with a Ki-67 labelling index of 7% (Figure 1c,e). Immunohistochemistry for ACTH was positive (Figure 1d), while other pituitary hormones, including GH and prolactin were not expressed within the tumour. Following surgery, morning ACTH was 6.0 pg/ml (1.32 pmol/l) and serum cortisol 4.1 µg/dl (113 nmol/l). Due to mild symptoms of adrenal insufficiency, the patient received low-dose hydrocortisone replacement (10 mg/day). In addition, the clinical signs of Cushing syndrome regressed over time, in keeping with disease remission. More than seven years after surgery, the patient remains free of signs and symptoms suggestive of a relapse of hypercortisolism.

As the patient's clinical phenotype was compatible with CNC, mutation analysis of the *PRKAR1A* gene was undertaken. This revealed a previously described heterozygous germline mutation in exon 2 (c.109C>T; p.Gln37Ter) that generates a premature stop codon ⁵, confirming the diagnosis of CNC in this patient. The mutation appeared to have occurred *de novo*, as confirmed by the lack of family history and negative genetic

testing in the patient's parents. *PRKAR1A*, which encodes for the type 1 alpha regulatory subunit of the protein kinase A, has been suggested to act as a tumour suppressor gene driving tumorigenesis in CNC patients, as previously demonstrated by loss-of-heterozygosity studies ⁷. Thus, we extracted DNA from the pituitary tumour of our patient and performed loss-of-heterozygosity analysis. By Sanger sequencing, we found a partial loss of the wild-type allele at the level of the mutation in the patient's pituitary tumour (Figure 2A). We then performed genotyping for two microsatellite markers (D17S942 – centromeric, and D17S789 – telomeric to *PRKAR1A*). While analysis of D17S942 was uninformative, genotyping for D17S789 confirmed the loss of the wild-type allele within the tumour (Figure 2B), with an allele peak height ratio of 2.2, in keeping with loss-of-heterozygosity.

Discussion

CNC is a rare genetic syndrome with a variety of clinical manifestations ²⁻⁴. Inactivating germline mutations of the *PRKAR1A* gene represent the most common cause of CNC. These mutations can be inherited in an autosomal dominant manner and disease penetrance is almost complete. However, only approximately 70% of CNC patients have a positive family history, whereas the remaining cases appear to be caused by *de novo* mutations ^{3, 5}, as in our patient.

CNC patients can develop several endocrine manifestations, also including pituitary disease. The most common pituitary tumours in CNC are GH-producing and sometimes co-secrete prolactin, whereas corticotroph adenomas have not been previously reported ¹¹. While *PRKAR1A* mutations do not typically lead to corticotroph adenomas, we need to keep in mind that CRH receptors type 1 and 2 signal through

cAMP¹². In addition, at least one case of a corticotroph adenoma has been described with an activating mutation of *GNAS*, encoding the regulatory G-protein alpha subunit¹³. Such mutations, leading to the constitutive activation of cAMP production, are responsible for McCune-Albright syndrome and represent common somatic mutations observed in somatotroph adenomas^{14, 15}.

Although loss of chromosome 17 has been described as one of the most common chromosomal alterations associated with corticotroph adenomas¹⁶, mutations of the *PRKAR1A* gene have not been previously identified in patients with Cushing disease^{17, 18}. Our data support that the *PRKAR1A* mutation played a pathogenic role in the development of the pituitary corticotroph adenoma in this patient, as it is unlikely, although not entirely impossible, that, in addition to CNC, this patient had a coincidental corticotroph adenoma with a somatic loss at the 17q24 locus.

While up to 60% of all CNC patients develop PPNAD and a significant proportion presents with clinically overt ACTH-independent Cushing syndrome^{2, 4, 5}, to our knowledge, this is the first published case of a CNC patient with Cushing disease due to a corticotroph pituitary adenoma. In our patient, concomitant PPNAD as the reason for Cushing syndrome was ruled out on the basis of biochemistry and remission of Cushing syndrome after resection of the pituitary adenoma. In a previously reported case of a CNC patient with PPNAD, factitious elevation of ACTH levels has been described, possibly caused by interfering antibodies with ACTH-like activity¹⁹. However, such possibility seems unlikely in our patient, considering the results of the IPS showing a significant central to peripheral ACTH ratio and the significant drop of the ACTH levels following transsphenoidal surgery. We are aware, however, that our patient has an increased risk for developing Cushing syndrome secondary to PPNAD during the course of his life, given the high frequency of PPNAD in patients with CNC.

In summary, we provide the first evidence that *PRKAR1A* loss-of-function mutations can be involved in the tumorigenesis of corticotroph pituitary adenomas. Hence, pituitary-dependent Cushing disease should be considered in the differential diagnosis of hypercortisolism in CNC patients.

Methods

Histopathology and immunohistochemistry

The tumour biopsy specimen was fixed in 4.5% neutral buffered formalin, embedded in paraffin, cut at 5µm and stained with haematoxylin and eosin. Immunohistochemistry was performed with a streptavidin-biotin-peroxidase complex method. Sections were stained with monoclonal antibodies for Ki-67 and ACTH (Dako, Agilent Technologies, Carpinteria, CA) using a Dako AutostainerPlus Link automated immunostainer (Agilent Technologies). For visualization the Envision FLEX Plus Dako kit (Agilent Technologies) was used according to the manufacturer's recommendations.

Loss-of-heterozygosity studies

Genomic DNA was isolated from the patient blood (Illustra DNA Extraction Kit BACC2, GE Healthcare, Little Chalfont, UK) and pituitary corticotroph adenoma tissue (QIAamp DNA FFPE Tissue Kit, Qiagen, Hilden, Germany). An amplicon encompassing the *PRKAR1A* c.109C>T mutation was amplified by PCR using the following primers: 5'-GCACGCAGCCTTCGAGAAT-3' (forward) and 5'-CTCCAACCTCTCAAAGTATTCCCTG-3' (reverse). The PCR products were sequenced by Sanger sequencing under standard conditions. Paired blood- and pituitary tumour-derived DNA samples were genotyped for two microsatellite markers, D17S942 (centromeric to *PRKAR1A*) and D17S789 (telomeric to *PRKAR1A*). The

fluorescently tagged PCR products were run on an ABI3730xl (Applied Biosystems, Warrington, UK), and allele peak heights were measured using the Peak Scanner Software v1.0 (Applied Biosystems). The ratio of allele peak heights of the normal and tumour samples was calculated as follows: (peak height of normal allele 2/peak height of normal allele 1)/(peak height of tumour allele 2/peak height of tumour allele 1). A value <0.5 or >2 was considered in keeping with loss-of-heterozygosity, as previously suggested²⁰.

Author contributions

FWK, YW, SW, EK, FT, MiKr, AL and AG cared for the patient. DI and MáKo performed the loss-of heterozygosity studies. RH performed the immunohistochemical analyses. FWK, AG, DI and MáKo wrote the manuscript. All authors reviewed and edited the manuscript.

Conflict of interest statement

The authors do not have relevant conflicts of interest.

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Informed consent

The patient's written informed consent was obtained for genetic studies and for publication of this manuscript.

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Figure Legends

Figure 1. MRI images of the brain showing a left-sided pituitary microadenoma (arrows) on coronal **(a)** and sagittal **(b)** views. Histological and immunohistochemical staining of the tumour shows large adenoma cells with abundant basophilic cytoplasm (haematoxylin-eosin, **c**), robust expression of ACTH **(d)** and a Ki-67 labelling index of 7% **(e)**; 400x magnification.

Figure 2. Sanger sequencing for the c.109C>T PRKAR1A mutation and microsatellite genotyping show loss-of-heterozygosity in the corticotroph adenoma. A. Sanger sequencing showed partial loss of the wild-type allele in the tumour sample, suggesting loss-of-heterozygosity. B. Capillary electrophoresis analysis for the D17S789 microsatellite marker in blood- and tumour-derived DNA, showing reduction of the wild-type (**) allele compared to the mutant (*) allele in the tumour sample. The ratio of allele peak heights was 2.2, confirming the loss-of-heterozygosity.

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